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On the Antibiotic Activity of Mycobacteria and Nocardia

Preliminary Report on a Tuberculostatic Substance contained in Bacteria Cell

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Introduction

Although streptomyces is recognized as the richest source of antibiotics, the contributions to the antibiotic activity of mycobacteria are very scarce. Researchers engaged in the field of antibiotic investigation seem to have little attention to this organism. There remains, however, the problem whether the mycobacteria are really poor producers of antibiotics or the investigations on the antibiotic production of this genus have not yet been made satisfactorily.

The present investigation is undertaken to throw some light on this problem.

Experiment

1) Isolation of mycobacteria from soils and feces.

Collection of soil or feces samples were made both in 1953 and 1954. In the former case 236 strains of mycobacteria and 31 strains of acid-fast nocardia were isolated from 119 samples of soil and feces. Sixty one strains of mycobacteria and 21 strains of acid-fast nocardia were isolated from 24 soil samples in the latter case.

2) Antimicrobial activity of isolated strains on the glycerol agar plates.

Cross streak method on glycerol agar plates were used as the test method for the antimicrobial activities of isolated strains. *Micrococcus pyogenes aureus* Terajima,

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Escherichia coli, and *Mycobacterium avium* Choju were used as the test organism. *Candida albicans*, *C. tropicalis*, *C. krusei*, *Trichophyton interdigitale*, and *Achorion gallinae* were also used for the antifungal activity test.

The assay was repeated at least three times for each strain and the inactive strains were discarded when the length of inhibition zones was less than 5 mm.

Table 1. Antibiotic activities of mycobacteria and nocardia on glcerol agar plates.

	Number of strains tested	Number of strains active aginst		
		E. coli	Micrococcus pyogenes aureus	Mycobact. avium
Case 1* 267 strains	Mycobacteria 236	0	0	25
	Nocardia 31	0	0	0
Case 2* 82 strains	Mycobacteria 61	0	0	14
	Nocardia 21	0	0	4

* Case 1: strains isolated in 1953
Case 2: strains isoloted in 1954

Table 2. Antifungal activity of 162 strains of mycobacteria on glycerol agar plates (cross streak test)

Active against	Numbers of active strains
Candida krusei	27
Three species of Candidas	3
C. albicans and C. krusei	3
All Candidas and flungi tested	2
C. albicans	2
Any one species other than C. albicans and C. krusei	5
	42

Test organisms: Candida albicans, C. tropicalis C. krusei, Trichophyton interdigitale and Achorion gallinae.

Results of the investigation were summarized in Table 1 and 2. As seen in Table 1 mycobacteria and acid-fast nocardia seem to exert their antimicrobial activity mainly against avian tubercle bacilli and have no effect on routinely employed test organism, *E. coli* and *Microc, pyog. aureus*. Antifungal activity of mycobocteria were, however, not negligible as seen in Table 2.

It may be also worthy to mention that the activity of mycobacteria against avian tubercle bacilli on agar plates were complete in many cases; i.e., no growth of the avian tubercle bacilli were observed on the plate where the active strain of mycobacteria had been grown (Table 3).

Table 3. Length of inhibition zone of active strains of mycobacteria against avian tubercle bacilli.

Length of inhibition zone	Case 1*	Case 2*	
		Mycobacteria	Nocardia
5–10 mm	4	0	2
11–15	3	0	0
16–20	2	0	0
21–25	0	0	0
>26**	16	14	2
Total	25	14	4

* On case 1 and 2 refer to Table 1.

** No growth on plates.

Strains of mycobacteria isolated in the present study were divided into two main groups from tneir colony appearance. In *the first group* the colonies were raised with a nodular or wrinkled surface and irregular margin, had a tendency to become dry with age, cream to yellow colored, i.e., generally speaking, they had some resemblance to the colonies of tubercle bacilli; the colonies of *the second group* were, on the contrary, smooth, moist, glistening, and spreading, yellow to orange in color, had no tendency to become nodular with age, microscopically some strains produced palisade arrangements of cells indicating some relationships to corynebacteria.

Again when we classified the antitubercular strains of mycobacteria according to the above-mentioned two groups the interesting result was found that almost all the active strains belonged to the first group as seen in Table 4.

Table 4. Classification of active strains of mycobacteria from their colonial morphology

	1st group**	2nd group**
Case 1*	17	2
Case 2*	14	0

* On case 1 and 2 refer to Table 1.

** Colonial morphology of the 1st group resembles to that of the tubercle bacilli and the 2nd group to corynebacteria.

Note: Of the 25 active strains of mycobacteria in the case 1 19 strains were examined of their cultural and microscopical characters.

3) Activity of the fermented broth of mycobacteria against tubercle bacilli.

Strains which showed the activity against avian tubercle bacilli on glycerol agar plates were sown on 4% glycorol broth and incubated at 30°C. On the 5th and 7th day of incubation their activity against avian tubercle bacilli Choji were tested by the glycerol broth dilution method. The fermented broth was, in this case, sterilized by preservation at 100°C for 15 minutes or by filtration through bacterial filter (Chamberland L 3).

The potency of both filtrates were shown in Table 5. The strains which showed the low potency less than 10 dilution units were eliminated from the table. As seen in Table 5 the potency of the culture filtrates of 7 strains, i.e., No. 222, 226, 228, 230, 232, 238 and 240 were higher when they were kept at 100°C for 15 minutes than when passed through the bacterial filter. It has been shown, on the contrary, that 5 strains showed the more excellent activity when their fermented broth were passed through bacterial filter (from No. 211 to No. 292).

Table 5. Activity of culture filtrates of mycobacteria against avian tubercle bacillus.

Strains tested	Inhibition zone on glycerol agar plates	Potency* of culture filtrates when	
		kept at 100°C for 15 minutes	passed through bacterial filter
No.		dilution units	dilution units
222	No growth	80	<10
226	10	80	〃
230	20	80	〃
238	No growth	20	〃
228	〃	10	〃
232	15	10	〃
240	No growth	10	〃
211	No growth	<10	80
229	〃	〃	10
241	10	〃	10
247	13	〃	10
292	No growth	20	80
260	No growth	160	160
179	10	20	
180	No growth	320	
278	20	20	
279	14	10	

* The potency of the seventh day incubation

4) Extraction and purification of an active substance from the mycobacterial cell bodies.

As mentioned above some strains, e.g., No. 222 and No. 230 had a relatively excellent activity both on the glycerol agar plates and in the culture filtrate kept at 100°C for 15 minutes in spite of their low potency when passed through the baterial filter. This made us to assume that the active substance might exist in the bacterial cell bodies.

An attempt to extract an active substance from the cell bodies of No. 222 strain of mycobacteria was made by the following procedure. The stock culture of No. 222 strain was inoculated onto the 4% glycerol agar in Roux bottles and kept at

30°C for about 7 days. The bacterial cell mass harvested from the bottles were ground in a motar, then extracted with ether. The ether extract was concentrated *in vacuo* and the crude yellow-colored ether extract was assayed against avian tubercle bacilli and No. 222 itself (self-inhibition[2]), being the titers of 1,600,0000 and 200,000 dilution units, respectively.

The crude extract was washed with small amount of ethanol. The ethanol insoluble function was extracted with amyl acetate twice. The amyl acetate extract was chromatographed on alumina column. Then the column was developped with amyl acetate. When the fractionated eluent was evaporated *in vacuo* to dry the crystalline antibiotic appeared. This substance was the colorless, needle crystals and had the potency of 0.05 γ/ml against avian tubercle bacilli.

Unfortunately, further investigation became impossible because of the rapid decrease of potency in spite or the repeated single cell isolation technique.

The authors isolated the active strains of mycobacteria again in 1954 and is investigating the extraction and purification of the active substance by the same procedure described above. The details of the results will be reported later.

Discussion

Though the contributions to the antibiotic activity of mycobacteria are very scarce,[3][4][5] all of them agree with at the point that the activity of mycobacteria is mainly against tubercle bacilli and not or meagerly against routinely employed test organisms such as *E. coli* or *Micrococcus pyogenes aureus.*

The present investigation coincides with previous ones in this respect but it also suggests that the antifungal activity of mycobacteria is not negligible.

It is a interesting fact that only a limited group of mycobacteria of which colonies resemble somewhat to that of tubercle bacilli exerts the antitubercular activity. This may be helpful for the selection of antitubercular strains only by observing their colonial morphology.

The fact that the active substance against tubercle bacilli exists in the mycobacterial cell bodies may be one of the reasons why the preceeding investigators overlooked the antibiotic activity of mycobacteria. However, it may not be an exaggeration to say that the production of the antitubercular substance of mycobacteria decreases more rapid than the case of nocardia and streptomyces. All of the active strains of mycobacteria isolated in 1953 were no more than active after 6 months of isolation and it was in vain to attempt to recover the activity by the single cell isolation method. This may also be another reason of the infertility of the antibiotic investigation of this genus.

Summary

The antibiotic activity of 297 strains of mycobacteria and 52 strains of acid-fast nocardia were investigated.

It was found that the antibiotic activity of mycobacteria aud acid-fast norcardia were mainly against tubercle bacilli but not against *Escherichia coli* or *Micrococcus pyogenes var. aureus.* Their antifungal activity were also not negligible.

No growth of avian tubercle bacilli was in many cases observed on the plate where the active strains had been grown.

Almost all the strains active against avian tubercle bacilli had somewhat similar colony appearance to that of tubercle bacilli.

The potency of the fermented broth of the active strains was not so high and the active substance of which minimal inhibitory concentration against avian tubercle bacilli was 0.05 γ per ml existed mainly in the bacterial cell bodies. The production of the active substance of mycobacteria seemed to decrease rapidly.

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